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DECLARATION OF L. ALISON MCINNES UNDER 37 C.F.R. § 1.132	
Address to: Assistant Commissioner for Patents Washington, D.C. 20231	
Attorney Docket Confirmation No.	UCAL142CON 2046
First Named Inventor	N.B. Freimer
Application Number	08/976,560
Filing Date	November 24, 1997
Group Art Unit	1655
Examiner Name	L. Arthur
Title	*Methods for treating bipolar mood disorder associated with markers on chromosome 18p*

Dear Sir:

1. I, L. Alison McInnes, declare and say I am a co-inventor of the claims of the above-identified patent application. I directed others and personally performed the research leading to the invention disclosed and claimed therein.

2. I have read the Office Action dated April 24, 2001 in this application and understand that the Examiner has rejected pending claims 1-12 and 25-27 on the basis that the specification is not enabling for the full scope of the claims.

3. The data presented below show that, using techniques described in the specification, at least five new polymorphisms, including single nucleotide polymorphisms (SNP), were identified in the narrow interval on chromosome 18p described in the application, which polymorphisms are associated with bipolar mood disorder (BP). Thus, in addition to the polymorphisms already identified in the patent application, and using the guidance provided in the application, several additional polymorphisms were identified that are associated with BP.

ASSOCIATION OF POLYMORPHISMS WITH BP IN A NARROW INTERVAL ON CHROMOSOME 18P AS IDENTIFIED IN THE INSTANT APPLICATION AND CORROBORATED BY SUBSEQUENT WORK

4. The instant application provided data showing a positive LOD score for a D18S59 allele with BP in a pedigree analysis; and gave evidence of an association of D18S59 with BP in a population study. The instant application further showed a positive LOD score for a D18S476 allele with BP in a pedigree analysis and gave evidence of association in population studies. In a subsequent study of linkage disequilibrium (LD) on chromosome 18 in a population sample of 69 BP-I patients from the Central Valley of Costa Rica (CVCR), the same D18S59 allele was associated with BP-I. Escamilla et al. (1999) *Am. J. Hum. Genet.* 64:1670-1678; a copy of which is provided herewith as Exhibit 2.

5. Further genotyping of the 69 affected individuals using four publicly available microsatellite markers delineated a segment of maximal LD with BP-I, covering about 331 Kb. Evaluation of a larger sample (227 patients and relatives, and 26 independent control trios) using these markers showed continuing evidence of LD and haplotype sharing in this sample for this region. Escamilla et al. (2001) *Am. J. Med. Genet.* 105:207-213; a copy of which provided herewith as Exhibit 3.

6. Thus, the instant application provides evidence of association of at least two polymorphisms associated with BP. This association was corroborated by work published after the filing date of the instant application. These markers are in a narrow interval between SAVA5 and ga203 on chromosome 18p. Within this region, a segment of about 331 kb, and having maximal LD with BP, was further delineated.

AT LEAST FIVE ADDITIONAL POLYMORPHISMS ASSOCIATED WITH BP WERE FOUND IN THE PREVIOUSLY IDENTIFIED NARROW INTERVAL

7. Using techniques described in the instant application, at least five additional polymorphisms were identified that are located within the narrow interval between SAVA5 and ga203, and that are associated with BP.

8. As described in detail below, four new microsatellite markers, and 26 new single nucleotide polymorphisms (SNPs) were identified in the narrow interval on chromosome 18p. The results of LD analysis of these 30 new markers, as well as four previously identified microsatellite markers, are displayed in Table 1. Of

the 34 markers presented in Table 1, 16 showed association ($\lambda > 0$) with BP in at least one of the two samples. The p-value for five of these 16 markers was < 0.01. All five of these markers (PH84, PH205, PH202, PH208, and TS30) had estimates of λ near 1.0, indicating that virtually all affected individuals had at least one copy of the associated allele.

METHODS

Sample collection

9. Two samples were analyzed. In one sample, the patient sample was composed of 227 CVCR BP-I individuals (including the set of 69 patients from Escamilla (2001) that gave the original association evidence in 18p) and their available first degree relatives (total N=563). All affected individuals had at least two psychiatric hospitalizations with the first hospitalization by age 50. A second sample was comprised of these 563 individuals and a set of controls (52 unrelated parents of students recruited from the University of Costa Rica who were selected for CVCR ancestry [at least 5 out of 8 great-grandparents from the CVCR]).

Radiation hybrid and STS-content mapping of markers within the candidate interval

10. Genetic and physical mapping information was initially obtained from Whitehead Institute for Biomedical Research/MIT Center for Genome Research, Stanford Human Genome Center, GÉNÉTHON Human Genome Research Center, and the Cooperative Human Linkage Center. Radiation hybrid (RH) mapping was used extensively in the early phase of this study to resolve discrepancies in marker order between maps. Specifically, the 83 Stanford G3 radiation hybrid panel was used to map all genetic and STS markers available from public database as well as those developed specifically for the project. In addition to RH mapping, STS-content mapping using BAC (Bacterial Artificial Chromosome) clones from the region of interest was also used routinely to determine the marker order and to complete the BAC contig.

BAC library screening, end sequencing and contig building

11. Microsatellite and STS markers obtained from public databases were used to screen the human BAC library from Research Genetics (Huntsville, AL) by PCR or to the BAC library from Genome Systems (St. Louis, MO) screen by hybridization according to manufacturers' protocols. BAC DNA from positive clones was prepared, and sequences of the BAC ends were obtained by cycle sequencing the BAC DNA directly with vector primers T7 and SP6, respectively. PCR primers were designed from non-repetitive end sequences and used as STS markers to improve the physical map and the BAC contig construction. The

outlying markers from each side of the contigs were used to screen for overlapping BAC clones to extend the contigs.

Construction of randomly sheared libraries from BACs

12. BAC DNA was sheared to small fragments of desired size range using a nebulizer. After shearing, the libraries were constructed using established techniques.

Microsatellite and SNP marker development and genotyping

13. Microsatellite markers were generated by hybridizing oligonucleotide probes for di, tri, and tetranucleotide repeats to randomly sheared sub-libraries made from BAC clones using Quicklite non-isotopic enzyme induced chemiluminescent reagents from Lifecodes Corp. (Stamford CT) following the manufacturer's instructions. Positive clones were sequenced to identify microsatellite sequences and primers were then designed from flanking unique DNA sequence. Primers for amplifying STS markers were also designed using BAC end sequences, and random sequences available within the candidate interval when extensive sequencing of the randomly sheared libraries were done. Primer sequences are publicly available at PNAS Online.

14. We genotyped the 4 new microsatellites identified by us in sequencing the region. Primer sequences are available on request. Genotyping procedures for the microsatellites were performed using established techniques.

15. Single nucleotide polymorphisms (SNPs) were identified using SSCP (Single Strand Conformational Polymorphism) analysis of STS markers (all < 300 basepairs in length), using established techniques. We used four unrelated individuals to screen for each SNP. We genotyped the SNPs in patient and control samples using standard SSCP procedures.

Sequencing of the candidate interval and identification of the candidate genes

16. In the interval of < 3 cM, located within the SAVA5-ga203 interval, randomly sheared libraries prepared from BACs covering this region were sequenced at 10X coverage to discover all sequence information and identify all genes within the interval. More than 10,000 individual sequences from the region were compared by BLAST20 with sequences from publicly available databases and were analyzed using GRAIL21 to identify potential coding sequences. In addition, sequences were assembled using PHRAP 22,

23, 24 in a single DNA strand of ~331 Kb. The whole sequence was again analyzed using BLAST and GRAIL to aid in gene prediction. These data were displayed in ACEdb (data available from ncbi.nlm.nih.gov) to visualize predicted exons and their relationships to each other.

Statistical analyses

17. We applied a modified version of Terwilliger's likelihood ratio test of LD to the 4 novel microsatellites and 26 SNPs that spanned our 331 Kb candidate region. For each of these 30 markers we applied this test twice, once in the sample of 227 patients and their available relatives, and also with the addition of the independent controls to the 227 patients and relatives. This likelihood ratio test estimates a single parameter, λ, which quantifies the overrepresentation of an associated marker allele on disease chromosomes versus control chromosomes. λ is related to the common epidemiological parameter of population attributable risk. If the frequency of an associated allele on disease and normal chromosomes is given by p_D and p_N, respectively, then λ is calculated by $(p_D - p_N)/(1 - p_N)$. Only positive associations with disease are permitted, and λ ranges from 0 (under the null of no association) to 1.0 (all disease chromosomes carry the associated allele). Others have shown that λ is the most closely related to the recombination fraction with disease and less influenced by marker allele frequencies than other measures of LD. Because we do not know which chromosome of an affected individual harbors the disease locus, we incorporated a genetic model of disease transmission in the procedure of Terwilliger. Using this model also enabled us to employ data from additional family members other than parents, if they were not available. The same genetic model (mostly dominant with reduced penetrance) was used as in our previous LD papers and in the genome screen of the Costa Rican pedigrees described in McInnes et al. In this model one chromosome of the affected individual is used as a control chromosome. The use of a model is likely to increase the power of the test and the precision of the estimates of λ when the inheritance pattern is approximately known. Using simulated data, Terwilliger shows that his test is conservative.

RESULTS

Marker development and physical map

18. Based on our previous results (as described in the instant patent application; and in the publications provided herewith) we focused marker development and physical mapping efforts (including direct sequencing) in the <3 cM region between sAVA5 and D18S1231. Within this region we identified 4 new microsatellite markers and 26 SNPs to add to the 4 publicly available microsatellite markers already used

(see Exhibit 3). Based on the extent of haplotype sharing in pedigree CR001 and LD results from the previously used markers, we focused our detailed investigation on the region of about 331 Kb between PH33 and D18S1231 (although in public databases this segment is estimated as being 378 Kb in length, contig NT_011005). Using several sequence analysis tools and database mining procedures (see Methods, above), we determined that this interval contained six known genes (*CENTRIN, CLUL1, TYMS, rTS, YES1*, and *ADCYAP1*, ordered from telomeric to centromeric, with *TYMS* and *rTS* overlapping each other). This order differs in the public database (*CENTRIN, CLUL1, YES1, rTS, TYMS*, and *ADCYAP1*, with no overlap between *rTS* and *TYMS*). All of the genes except "clusterin-like 1 (retinal)" gene [*CLUL1*] have been well characterized previously. *CLUL1* was originally identified during a screen of a human retinal cDNA library for retina-specific genes. The function of this gene is not known; however Northern blot analysis reveals that it is highly expressed in retina with much lower yet detectable expression in several other tissues including brain, kidney and testes.

Genotyping results

19. We genotyped the 30 new markers in pedigree CR001 and in the CVCR patient and control samples. Results of the LD analysis for these markers (and the four previously available markers reported in ref 8) are displayed in Table 1 (provided herewith as Exhibit 4). Of the 34 markers presented in Table 1, 16 showed association ($\lambda > 0$) with BP-I in at least one of the two samples (that with 227 patients/relatives and that with 227 patients/relatives and the addition of 52 controls). The p-value associated with the estimate of λ was <0.01 for five of these 16 markers, and for four of the five markers the magnitude of association was greater in the sample containing the population controls. All five of these markers (PH84, PH205, PH202, PH208, and TS30), had estimates of λ near 1.0, indicating that virtually all affected individuals had at least one copy of the associated allele. The markers showing LD are clustered in the 19 Kb segment between exon 8 of CLUL1 and exon 1 of TYMS. This segment also contains the minimal region of haplotype sharing within CR001, and for each marker in this segment, the associated alleles seen in the population samples are the same alleles in the shared haplotype in CR001 (last column in Table 1).

SUMMARY

20. The data presented herein extend the findings described in the instant patent application. The patent application provided evidence, from both pedigree analyses and population studies, that a number of polymorphisms, including a 154 bp allele of the microsatellite marker D18S59 and a 271 bp allele of the microsatellite marker D18S476, are associated with BP. The patent application described how to identify additional markers, and how to determine whether such markers are associated with BP.

21. The data presented herein show that, using techniques described in the patent application, several new polymorphisms, located in the previously identified interval and associated with BP, were identified.

CONCLUSION

22. Those in the field, given the guidance in the instant patent application, could identify additional polymorphisms associated with BP.

23. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title XVIII of the United States Code, and that such will false statements may jeopardize the validity of the application or any patent issuing thereon.

OCT 9 2001
Date

L. Alison McInnes

Enclosures: Exhibits 2-4

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